Journal of Pharmaceutical and Scientific Innovation
www.jpsionline.com
Research Article

PEPSIN ASSAY ONE OF THE EASIEST APPROACH FOR PRESCREENING OF HIV-PROTEASE INHIBITORS

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Received on: 04/12/12 Revised on: 25/02/13 Accepted on: 28/02/13

ABSTRACT
As HIV is quickly becoming one of the world deadliest viruses, one of the biggest problem in curing AIDS is as HIV can easily develop resistance against the provided drugs. HAART (Highly active antiretroviral therapy) is the only one hope for the AIDS patients till now, which can increase the survival period of the AIDS patient by sustaining the viral load below 50 copies/ml. in blood serum, in which we use combination of three to five drugs targeting different stages of replication cycle of HIV. HIV-protease inhibitors are the indispensable part of this therapy. There is a great need of screening of anti-HIV agents from chemical as well as natural resources. But this process is not so easy because it needs huge amount of money for required chemicals, as well as highly sophisticated labs. Here we have developed a substitute of this problem unto some extent, i.e." Pepsin Assay", it is a substitute of HIV- protease, both of them belongs to same Aspartyl family of enzyme & share same signature sequence at the active site. So we hope that development of this assay will enhance the screening programme of HIV-protease inhibitors.
KEY WORDS: HIV-protease inhibitors, HAART, Pepstatine A, STI, AIDS, CD4+ Cells.

INTRODUCTION

A number of laboratories in the world are actively involved for the investigation of anti-HIV agents that interfere with different stages of HIV replication cycle. There are still no cure & no vaccine for AIDS. But treatment with a combination of two nucleoside analogues and a protease inhibitor (triple or combination therapy) has been in use since 1994 and remarkably successful in halting virus replication and the progression of AIDS. There are many evidence of research in support of efficacy of combination therapy[1-4]. This therapy is now known as highly active antiretroviral therapy (HAART), however viral level in plasma reaches up to an undetectable level under a successful triple therapy (i.e. upto < 50 copies/ml.), still HAART unable to clear the virus completely from the AIDS patients. There is a latent virus present in the resting CD4+ memory cells and other reservoirs as well, so the treatment must be continued without a long break, long term break cause the virus to rebound within a few days to its original level, but prolonged exposure to a particular drug cause the development of the resistance against the provided drug, some researchers suggest that short term break in treatment almost always lead to viral rebound that can be re-suppressed with HAART; immune response to HIV improves. This therapy also called Structured Treatment Interruption (STI)[5]. There are many other problems with the conventional chemotherapy provided for the treatment of AIDS, the clinical trials for the administration of these compounds to the AIDS patients revealed serious side effects. So there is a great need of development or screen out different compounds having anti-HIV activity with minimum toxic effects, which relates to different pathways of HIV infection, should prove important to prevent disease progression. It seems that a better strategy to find novel antiviral agents with less cytotoxicity is to look for natural substances. Many reviews on medicinal plants show the importance of natural products for curing many highly threatening diseases such as cancer, AIDS etc. Singh I.P. et. Al. reviewed natural anti-HIV products targeting different stages of HIV life cycle[6].

Over the last decade, antiviral researchers have also turned too many of the traditional folk medicines, invariably a 'cocktail' of natural products, to uncover the scientific basis of their remedial effects. Recently review published plant-derived anti-HIV[7-9] compounds, which serves to underline the fact that selected medicinal plants with HIV-inhibitory activity are widely distributed in nature[10-11].

HIV-protease is the indispensable part of HAART therapy, as it plays a significant role in the HIV replication cycle. HIV protease was first suggested as a potential target AIDS therapy by Kramer *et.al.* 1986[12]. After that it was shown a frame shift mutation in the protease region of the pole-gene prevented the cleavage of the Gag-Pole polyprotein precursor, which is the essential process for the maturation of structural & functional viral protein. Blockage of HIV protease leads to the formation of immature non-infectious virions. Compounds having ability to inhibit this protease have been studied intensively during the last decade and numerous reports have been published, Alterman, M 2001gave a comprehensive summery of design and synthesis of HIV-1 Protease inhibitors[13]. Saquinavir was the first approved protease inhibitor & has been in clinical use since 1995[14], presently there are six clinically approved protease inhibitors in the market available, although the inhibitors in the market are highly selective they induce side effects such as lipodystrophy, hyperlipidaemia, insulin resistance and emergence of the resistant mutants upon prolonged use of a particular drug therefore there is probably be a constant demand of new HIV protease inhibitors. Now the medical science community turned towards the extracting natural resources like plants[15-17], herbs[18], marine algae[19] and many other organisms for those bioactive compounds by which we can cure so many human disorders and pathogenic infections including AIDS and cancer. Here we developed a indirect approach for screening of HIV protease inhibitors from natural products.

HIV-Protease

Enzyme such as HIV-protease, natures own catalysts, proteases are a divers class of enzymes that catalyze the cleavage of peptide or proteins. Based on the presence of the characteristic signature amino acid sequence Asp-Thr-Gly. It was suggested by Toh *et al* in 1985 that protease of HIV might be belongs to the family of Aspartic proteases[20]. This was confirmed through Pepstatine A inhibition, an aspartic protease selective inhibitor[21] and by sit directed mutagenesis of the active site Asp-25, which led to abolition of the catalytic activity[22-23]. the aspartic proteases are the well characterized group of enzyme that can be found in vertebrates, plant, in addition to in fungi, example of proteases from the Aspartic protease class are- Pepsin, Cathepsin D, Renin, Chymosin, Penicillopepsin and Rhizopus pepsin; which all are two domain enzyme with more than 300 residues in length and contain the Asp-Thr-Gly sequence in each domain that form the active site, which effectuate the cleavage reaction since the HIV protease sequence is no more than 99 amino acids and contains only one one the required triad Asp-Thr-Gly it was suggested that the active form of the HIV protease was a homodimer of 198 amino acids[24]. This hypothesis was later confirmed by X-ray crystallographic determination[25-26]. As both HIV-protease & pepsin share the signature sequence Asp-Thr-Gly, an overall similarity of primary structure, inhibition by pepstatine A and are inactivated by mutation of the putative active -site aspartates therefore these all data suggests that pepsin may be a representative for the HIV protease in the aspartic group[27]. So pepsin assay could be used for pre-screening of HIV protease inhibitors. Maria del *et.al* 2004 also used the assay system to screen out HIV-protease inhibitor from Rapseed protein hydrolysate[28]. A detailed Computational Studies on HIV-1 Protease Inhibitors was performed by Wesley Schaal[29].

MATERIAL & METHODS

Chemical required

Enzyme Pepsin (Himedia), Hemoglobin (Himedia), plants extract, Sodium Acetate tri hydrates (Qualigens), Sodium Chloride (Himedia), Acetic acid, Tri Chloro Acetic acid (TCA) (Sdfine) and experimental plant material.

Instruments required

Spectrophotometer - Karry 100, Sigma table centrifuge for 14000 rpm, Rami for 8000 rpm, Micropipette 5-40 µl, 40-200 µl, 200-1000 µl, Eppendorff (1.75ml), Mixer grinder, Mortar pestle, electronic balance.

Plants extract preparation

Many plants were selected for our study, out of which *Aspargus recemosus*, *Zingiber officinele*, *Aloe barbadensis* plants give more significant results. Different parts of fresh plant material were taken, washed properly under tap water, 5 gm of each were taken, Cut it into small pieces, Ground it properly in mortar and pestle, 10 ml of DW were added to get fine paste, Centrifuge for 30 min. at 8000 rpm. Supernatant were collected & pallet were discarded and supernatant was used as a crud extract of plant material.

Enzyme Pepsin activity inhibition Assay: - pepsin have a quite close resemblance in proteolytic activity with HIV-1 protease one of key enzyme of HIV-1 life cycle as both of them belongs to same Aspartate enzyme family 28. So here we use this enzyme as a substitute of HIV-1 protease to check out anti HIV activity of plants extract.

Procedure

We have done slight modification in this assay system as mentioned by Maria del et.al. in pepsin assay the ref paper, we have taken almost double quantity of all constituents as well as reaction mixture and here we use crud plant extract in place of known amount of partially purified plant extract as used in previous works.

For that assay we have taken 50µg pepsin, 800µg hemoglobin and crud plant extract in 500µl of reaction mixture. Allowed to incubate for 20 min. at 370C, 700µl of 5% TCA was added to stop the reaction, after few min., centrifuge at 14000 g for 5 min. and supernatant was collected and O.D. was taken at 280 nm.

Separate blank were used for both positive and negative control as well as for sample. For positive control we have taken enzyme and substrate and follow the above procedure, and for negative control we have taken pepstatin as a well known inhibitor of both Pepsin and HIV-Protease. And for testing sample we have taken extract in place of pepstain. We have taken four plants for that study which gives almost same results with several repetitions, so this assay gives reproducible results.

Several experiments were performed to get optimum activity of enzyme:

Parameter - optimum range
pH - 2- 4
Incubation temperature - 37 0 C
Incubation period - 20min.
Centrifugation - 14000 g.
Reaction volume - 500 µL

Scanning of Hemoglobin solution was performed in the range of 150 to 650 nm. Gives three peaks, first at 206 nm, second at 280 and third at 410 nm (figure-1). Highest peak at 206 nm denote most probably the presence of large number of peptide bonds in Hemoglobin protein, peak at 280 must be due to the presence of tyrosin and tryptophan amino acids and peak at 410 nm might be due to the presence of Heam group in that protein. But here we choose peak at 280 nm for our experimental observations.

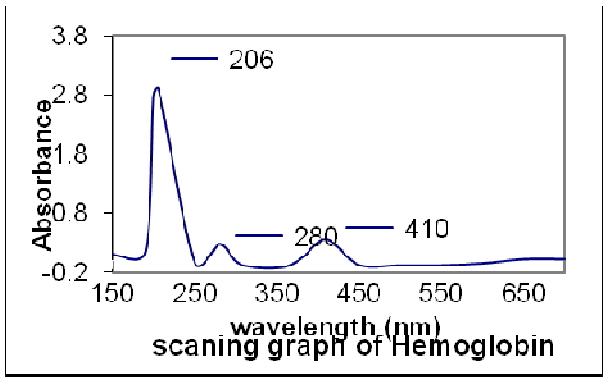

Fig. 1: scanning of Hemoglobin shows three peaks. At 206 at, at 280 and at 410 nm

We performed another experiment for pepsin enzyme kinetics; here we got a straight line (Fig.-2) while increasing concentration of hemoglobin within the range of 200- 800 µg above that a drop in slop had been observed and keeping enzyme conc. (50 µg) Constant in a 500 µl of reaction mixture.

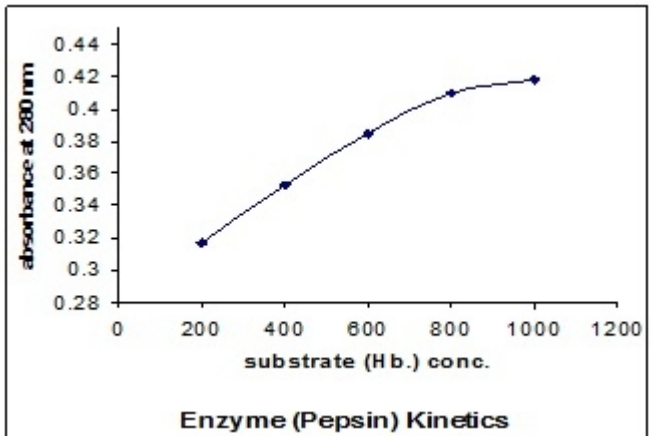

Fig.2: Enzyme (Pepsin) kinetics keeping enzyme conc. Constant.

Finally we performed enzyme inhibition assay, here we take three extract of plant sample that is Asparagus sp., zingiber sp. and alovera sp. and activity of enzyme were observed. We have also taken papestatin A as a negative control.

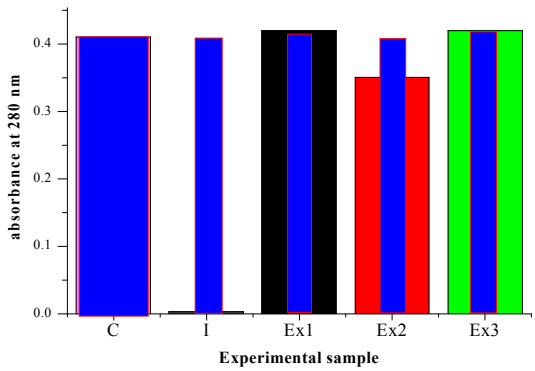

Fig.-3 Blue color bar (C) shows activity of enzyme in the absence of any inhibitor, negligible bar (I) shows maximum inhibition in the presence of natural pepsin inhibitor (Papestatin A). Ex1, Ex2 and Ex3 bar shows enzyme activity in the presence of different plant extract i.e. *Aspargus recemosus*, *Zingiber officinele*, *Aloe barbadensis* respectively, and middle blue bar shows the comparison of enzyme activity in the presence of plant extract with control simultaneously.

RESULT AND DISCUSSION

The purpose of this work was to develop an assay system for prescreening of HIV-1 protease inhibitors form crud plant extracts, as the previous research shows that pepsin had great similarities with HIV- protease in structure and function as well, so we can use it as a cheapest substitute of HIV-protease. Many research groups had been developed different kind of assay system with pepsin. Here we set different parameters according to our requirements. Basic mechanism behind this assay[30] is that when we allowed reaction by mixing enzyme and substrate in reaction vial left them for incubation for few minute then enzyme pepsin cleaves substrate hemoglobin into small pieces after incubation we added TCA (Tri Chloro Acetic acid) to stop the reaction simultaneously the large protein particles also get precipitated but the digested protein i.e. Smaller peptide or cleaved product of enzyme is still soluble in the reaction mixture and we can remove undigested precipitated part leaving digested part in the reaction mixture by centrifugation at 14000 g. and we take absorbance at 280 nm, which comes due the presence of tryptophan and tyrosin amino acids incorporated into the soluble peptide of digested Hemoglobin. In our experiment we have taken three crud plant extract to check the inhibitory effect their extract in this assay system, and we observed slight inhibitory effect with crud extract of zingiber officinale while the enzymatic activity of pepsin were not affected by the other (Aspargus recemosus, Aloe barbadensis) extracts. We have taken paptsetin A as a negative control, which natural inhibitor of this enzyme show approximately 99% activity inhibition of this enzyme. The results are reproducible and I hope that this assay system will encourage the prescreening programme of HIV-1 protease.

ACKNOWLEDGEMENT

The author is very grateful to Professor G.S. Randhawa, Head Department of Biotechnology, IIT ROORKEE, INDIA, as they provide technical assistance during my study, and also to Mr. Manish Rana, for their kind support.

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	Website http://www.jpsionline.com

How to cite this article:
Singh K.P., Kumar A., Prasad R. Pepsin assay one of the easiest approach for prescreening of HIV-protease inhibitors. *J Pharm Sci Innov*. 2013; 2(1): 53-56.